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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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III. Results of the method technic modification - Stahlin, V.H

(26)

The method described, which we term balanced method, was employed with a diluted strain of Bac. anthracis. It produced a united counting objective insofar as it builds no long threads but relatively short rods on a definite culture medium, which are usually found single or in chains with at the most from two to three links. The bacteria were strained on an agar culture medium and were then suspended in tap water with 1% formaldehyde. 13 preparations were produced and 35 sample fields were counted; an average of $6,51 \cdot 10^7$. cells were obtained per cubic centimeter. A number of control counts in a "Helber" bacterial count chamber (produced by the Hellige Co.) which is 0,02 mm deep, resulted in a $6,10 \cdot 10^7$ average. If we consider the average value between the two count chamber results as the true value, the maximum deviation of a single value obtained by the balanced method, from the true value is 27% and the average square deviation of the balanced method results from their own average is $0,95 \cdot 10^7$ or 14,5%. An example of a count is given here. The counted sample fields were arranged in 5 horizontal and 7 vertical rows and gave the following cell counts (Tab. 1)

Table 1. Cell count in 35 sample fields, which are equally distributed throughout the preparation

6	6	7	10	17	6	8
16	5	11	20	12	13	11
8	12	13	6	10	10	10
7	7	19	9	10	8	6
8	10	6	11	7	5	3

$g = 4,4$
$m = 333/35 = 9,51$
$F = 520$
$G = 1/57$

$$Z = 1000 \cdot \frac{9,51 \cdot 520 \cdot 57}{4,4}$$

$$\therefore = 6,41 \cdot 10^7$$

If we take the average from the 9 central sample fields in Fig. 1, we note that it is much higher than the average of the remaining 26 fields, i.e., 12,2 as opposed to 8,7. This intensifying of cell numbers near the center was met as a rule in all preparations produced according to the method described above, and may well rest on the fact that a number of cells remain attached to the upper or lower glass surface while the liquid is spreading. Furthermore we noticed an identical occurance in the Helber-chamber. By not filling the latter from the side, but by placing the drop on the chamber floor and then placing the cover glass over it, we found that the values were much higher when the drop was placed on the chamber net cross section, then when deposited at a few milimeters distance.

A suspension of micrococcus pyogenes (var. aureus) was then also counted twice according to the described balanced method; the obtained values were $14,4 \cdot 10^7$ and $16,1 \cdot 10^7$ cells per cubic centimeter, while the average from 2 Helber-chamber counts gave $14,06 \cdot 10^7$. Further straphylococcus counts were discontinued by this method, as it became clear that an extremely unequal cell distribution takes place with staphylococci, that the cocci are so deep in one or more areas of the preparation center that they defy counting; the periphery is then correspondingly poor in cells. The cocci of the Micro. pyogenes strain in question, had a very limited tendency to bunch-up, most cells lay alone or in pairs after agitation of the suspension by hand, very few bunches appeared which could not be counted. The mentioned bunching-up of the cocci near the preparation center is therefore hard to understand, the more so, since a corresponding test showed that cocci suspended in water with 1% formaldehyde had a lesser tendency to remain attached to the glass

than anthrax bacilli.

a. First modification. So as to circumvent this calculation difficulty, we placed the cover glass on the instrument stand to weigh it, then brought a drop of bacterial suspension into contact with the side of the glass, so as to distribute the liquid under the entire cover glass through capillarity; the liquid remaining on the edge was eliminated with blotting paper, it was weighed and the preparation was sealed off with vaspar. Much effort has been concentrated on construction of counting chambers in a manner to allow filling from the side by pouring the liquid, as this gives better results than placing the drop on the actual area to be counted. Staphylococci in such a preparation have proven equally distributed and easily counted. 5 preparations were made from one suspension and counted; the main deviation from the average was 9.3% and the standard deviation 7.9%. No comparison with a counting chamber was made in this series. The disadvantage of the operation just described stems from the fact that one has little influence on the layer thickness, since the quantity of liquid flow between the carrier and the top glass may vary considerably due to capillarity; as a result the layer is sometimes too thick, at other times too thin and is often different at various spots in the preparation.

b. Second modification. So as to also meet this circumstance, the cover slide was attached to the object carrier, before placement of a drop of solution. The preparation is produced in the following manner: a very small quantity of vasolene is placed at each corner of the upper surface of a cover slide by means of an instrument, a wire for example. The cover slide with the vasoline facing down is then pressed onto an object carrier. Vaspar is then applied to two opposite edges of the cover slide in a manner to com-

pletely seal off the space between the cover slide and object carrier; the preparation is then weighed on the analytical tare balance and a drop of the suspension to be tested is placed on one of the open edges of the cover slide; the remainder is cleaned off after the liquid has spread out under the entire cover slide and the preparation is again weighed. The two open edges are also sealed with vaspar after the weighing. It becomes easy after a while to seal the cover slide by means of a vaspar covered wire, so as to avoid smearing vaspar on the cover slide surface; this is achieved most easily by first placing the vaspar on the object carrier at a short distance from and parallel to the cover slide and then to let it flow on to the slide edge by passing over it with a clean, hot wire. With this method the layer thickness may be influenced up to a point, by exerting pressure on the four corners of the cover slide, when it is attached to the object carrier by means of vasoline.

Cells of various sizes were counted and the data compared with counts from the Helber-chamber, after this second modification, which in most cases appeared the most suitable of the 3 methods described. (Tab. 2). The suspensions were correspondingly diluted for the cahmber counts.

One may conclude from Table 2 that, assuming the chamber count value to be correct, the balance method in general gives approximately correct values for small bacteria (micrococcus, Pseudomonas); this also applies for Erythrocytes. On the other hand the vertical deviations in Bac. Anthracis are considerable, and a strong dependence of error on the layer thickness may be noticed here; the smaller it is, the stronger the vertical deviation of the attained value from the true value. The size of the anthrax bacilli cannot be held completely responsible for these deviations, for they should then

also appear with erythrocytes; the fact that the anthrax bacilli under consideration show a definite tendence to adhere to upper glass surfaces might possibly play a part.

Table 2. Results of repeated counts of various suspensions according to the balance method and the Helber chamber. Germ count data per cm^3 in ten million units. (D = average)

Susp. Nr.	Particle	Results		Average layer thickness in u
		Chamber count	Balance method	

Suspension Nr. 5 was mainly concerned with capsulated hay bacillus individuals. The great variations among the chamber count results is very noticeable; the standard deviation is approximately 26%, while it should be approx. 6% considering the Poisson-particle distribution in the suspension (Abbe, 1878, Student, 1906/07), as an average count of 288 cells was attained per computing chamber.[1] The standard value deviation of single counted squares within a computing chamber was approximately equal to the square root of its average value, as is to be expected in a Posson-distribution. The deviation growth in the single chamber counts may well be partly caused by the fact that the capsulated bacilli evidence a very slow rate of sedimentation; the count is therefore slowed down, as the entire light must be investigated. In the meantime evaporation sets in at the edge, manifested by a light streaming within the chamber. Evaporation and mainly the current resulting from the latter, naturally influence the result when they are limited in nature. Only a limited number of cells had settled on the bottom after the counting chamber had been left in the damp chamber for several hours. The differences between the separate results for suspension Nr. 5 are greater than expected even with the balanced method; the standard variation consists of 12,5% instead of 5% (an average of 402 cells were counted for every preparation). However the discrepancy between the expected and actual value of the average square variation is not as great as that of the same suspension count taken in the Helber-chamber.

1. See Sect. V for the calculation of the expected standard percentage deviation for the number N of a counted particle.

So as to see whether a difference exists in the preparation cell contents when the suspension is taken by pipe or pipette, 6 test lots of anthrax bacillus suspension were taken by means of a pipe or 6 with a pipette and they were counted in a Helber chamber, each time resulting in 400 cells. The lots taken with a pipe gave a cellular average of $4,87 . 10^7$ per cubic centimeter with a standard variation of 4,8%; the pipette tests resulted in a cellular average of $5,48 . 10^7$ per cubic centimeter and a standard variation of 29,8%. The difference between the two averages is considerable and the possibility that the two originate in different totalities is of approximately 9% according to the t-test (Fisher, 1936). A similar test was then conducted with a suspension of human erythrocytes in sodium citrate and formalin, where the one half of the Helber-chamber was filled with the solution by means of a pipe, and the other with a pipette, in such a manner as to eliminate communication. The preparation was sucked into the center of the plate in the Reagen glass, as in the case of the Bac. anthracis suspension. Naturally care was always taken to homogenize the suspension well before every test, by the presence of Newton rings after every filling.

100 squares of 1/400 mm^2 were counted during every erythrocyte count. The average of the erythrocytes found with the 6 pipette tests was 326.1, while the 6 pipe tests had 332.8. This difference naturally still falls within the area of chance. The preparations taken with a pipe show a higher degree of variation than those taken with a pipette, in contrast with that of anthrax, namely a standard deviation of 18% as against 11,8%. We should not, here, enter into the various causes of this discrepency, however the results may indicate that a considerable possibility of error must still be expected in chamber counts.